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13. ABSTRACT *(Maximum 200 words)*

We are using a novel approach developed at our Institute which makes use of random peptide libraries expressed on the surface of filamentous phage in order to identify in vivo peptides that may confer preferential homing properties to cancer cells for bone tissue. This approach is also complemented by in vitro panning using immortalized bone marrow stromal and endothelial cells as well as by expression cloning of cDNAs expressed differentially by metastatic breast cancer cells. To-date we have identified 10 peptides that appear to home to bone marrow by in vivo selection and one additional peptide that was positive in our in vitro selection system. Furthermore we have succeeded in immortalizing eleven different bone marrow cell lines which will be useful in our expression cloning experiments. These experimental approaches may lead to the identification of peptide sequences effective in blocking metastasis and serve as therapeutic compounds. This may lead to uncovering the basic mechanisms of bone metastasis by cancer cells which remains today one of the fundamental unresolved problems in tumor biology. Furthermore, identification of bone-specific homing sequences would enable the design of vectors to be used in gene therapy of genetic diseases affecting bone.

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FOREWORD

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5. INTRODUCTION

Bone is the most common site of metastasis of breast cancer cells and approximately 70% of patients with breast cancer have skeletal metastases at the time of autopsy. In spite of the frequent occurrence of bone metastases and their grave consequences to the patients, the mechanisms that favor bone as the site for metastasis of breast cancer cells are not known. There is no information available about the homing molecules or homing receptors that may be participating in this process. We are using a novel approach developed at our Institute which make use of random peptide libraries expressed on the surface of filamentous phage in order to identify peptides that may confer preferential homing properties to cancer cells for bone tissue. Identifying such a peptide(s) would immediately pave the way to isolating ligands and receptors that mediate homing and metastasis of breast cancer cells to bone. The successful identification of such peptides would facilitate the screening of cDNA libraries from breast cancer cells to clone the sequence-containing molecules and, subsequently, their receptors on the surface of endothelial cells. These peptide sequences may also be effective in blocking metastasis and thus become useful novel molecular therapeutic compounds. This experimental approach may lead to uncovering the basic mechanisms of bone metastasis by cancer cells which remains today one of the fundamental unresolved problems in tumor biology. In a more general application, identification of bone-specific homing sequences would enable the design of vectors to be used in gene therapy of genetic diseases affecting bone.

6. BODY

The approved tasks for this project included:

1) To generate and screen random peptide libraries in vivo to identify sequence motifs homing specifically to bone.
2) To ascertain if the peptide sequences exist in the context of full length cDNA molecules present on metastatic breast cancer cells
3) To identify and clone the receptors on the surface of bone marrow endothelial cells that are recognized by the homing peptides.

During this past year we have concentrated on Task 1. I will describe the results obtained, some of the difficulties encountered and some alternative methods and approaches which we have implemented successfully to advance our work.

Phage display:

Initially we used mixture of 7 random peptide libraries, CX5C, CX6C, CX7C, CX9, X4YX4, CX3CX3CX3C, CX3CX4CX2C, which had proved to be of good quality for in vitro panning in the hands of our collaborators in Dr. Ruoslahti's laboratory. Ten to the eleventh units of the library mixture was injected into the tail vein of 7-week old female Balb/c mice and allowed to circulate for 3 min. After snap freezing the mice in liquid nitrogen, bone marrow tissue was collected by flushing out the femurs, washed and incubated with host *E. coli* K91 kan. A total of 160 single colonies of infected bacteria were isolated, expanded individually and then pooled. The phage were purified and reinjected. We repeated 3 cycles of injection and recovery of phage. Two clones appeared 3 times and one clone appeared twice in 47 clones sequenced. We continued to run 3 additional cycles of injection and recovery and obtained sequences of 36 additional clones. We also performed three additional cycles using perfusion instead of snap freezing in order to minimize the destruction of the tissues and the possible leakage of phage into the extra vascular cavity in the bone marrow and sequenced an additional 36 clones. In order to find clones which might specifically recognize bone marrow endothelial cells, we incubated phage of the 3rd cycle with bone marrow stromal cells which are enriched in endothelial cells under supplement of endothelial growth factor and heparin and 35 clones that bound to the cultured endothelial cells were sequenced. Analysis of the sequences obtained by the different methods indicated that some of these peptides displayed homologies to interesting proteins such as coagulation factor VIII, entactin, opioid-binding cell adhesion molecule, insulin receptor and $\alpha 4$ $\beta 1$ integrin and 10 different peptide motifs were chosen for further characterization. Localization of these clones when injected individually, however, was not possible by immunohistochemistry with antibody against gp8 coat protein of M13 phage. We think this was may be due to low affinity binding or too low number of target molecules. In our second set of experiments, the slightly simpler libraries, CX6C, CX7C and CX10C, were mixed with non-infective fUSE5 to block non-specific interaction. The mouse was incubated for 60 min. and then perfused. After 3 cycles of injection and recovery from about 280 single clones, 4 clones appeared twice in 35 sequenced. We are currently attempting to stain tissue sections to assess the site of localization of these clones in the bone marrow.

In both sets of experiments, the enrichment of clones was not as high as we had originally expected. We found that bone marrow tissue retained an extremely high number of phage compared to the numbers per weight in other organs, such as brain, kidney and lung. This indicates that there is non-specific capturing of phage in the bone marrow. In order to check if the phage enter the extra vascular cavity and bind to hematopoietic cells, and/or if phagocytotic cells, such as macrophages and stromal cells, are responsible for trapping the phage, a large excess of non-selective phage were injected, and perfused after incubation of 1min., 3min., 60 min. and 24 hrs. Immunohistochemistry showed positive stainings mainly in the endothelial cells and not in extra vascular cavity, suggesting that most of phage that we recovered were at least specifically associated to the endothelial cells. Data base search of these peptides through Bliz data base (UK) showed some homologies to known proteins, such as insulin receptor and IGF-I receptor within the region of binding to a subunit of IGF-I. This phage was injected into mice together with standardizing phage, which has wild type gp3 and carries ampicillin resistant gene. Recovery of the phage clone from several organs was compared to the wild type standardizing phage, and following numbers were obtained per 1.0 standardizing cone; bone marrow 9.7+2.4, brain 1.5+0.21, lung 4.0+ 0.41, pancreas 3.5+0.28. We also injected this IGFR clone mixed with 4 times of non-selective phages inject into a mouse and recovered phages from bone marrow and brain. From bone marrow, 7 clones were the IFGR clone out of 12 clones sequenced, while from brain only 2 clones were the IGFIR clone out of 10 clones sequenced. Thus, recovery from bone marrow was 84% and that of brain was 20%, which number is close to the original amount of the clone, 25%. We have not proven that this clone binds to IGF-I and insulin, and it may recognize some unknown molecule, since it did not seem to home to pancreas, where insulin is abundant. IGF-I is, however, the third richest growth factor in bone matrix. Also this clone has homology to integrin $\alpha 4$ $\beta 1$(VLA-4), which is a receptor of VCAM-1 expressed on bone marrow endothelial cells and some stromal cells. These results appear very encouraging, and we will pursue this peptide as a possible specific homing molecule to bone.

Panning using bone marrow stromal primary cells

As an alternative to using the phage approach in vivo, we decided to extend our experimental approach to include panning in vitro using bone marrow stromal cells, since adhesive interactions between bone marrow stromal cells and other types of bone metastatic cancer, such as melanoma and prostate cancer, have been reported. We do not know which cell type may play an important role in the initial settlement of breast cancer cells in bone tissue. It is possible that the endothelial cells and stromal cells are involved in this process by different mechanisms. Therefore, it was deemed important to work with endothelial cells and stromal cells separately in these experiments. For this purpose, we have begun to generate immortalized cell clones of both endothelial and stromal cell that retain the ability to bind to bone metastatic breast cancer cell, MDA-MB-231. After the obtained clones are tested for binding to the MDA-MB-231 cells and characterized individually they will be used for the panning experiments

Primary cultures of bone marrow derived-endothelial cell were prepared from tibias and femora of 10 female 6-week old mice, and cultured in 3.5 cm diameter plastic dishes with ECGF (Endothelial Growth Factor) for six days in order to enhance the proliferation of the endothelial cell population. The cells were tested for the expression of TNAP (tissue non-specific alkaline phosphatase) and binding to MDA-MB-231 cells. The rest of the cells were stored in 100% ethanol at -20°C. The cells in a dish were washed with serum free DMEM and incubated with 100% FCS for one hour for blocking. Phage library with peptides of CX7C and that of X7 were added into FCS at concentration of 2.8×10^{11} cfu/ml for each library, and then incubated with the cells for one hour. After washing for one hour with 10 changes of serum free media, 2M NaCl were added to elute bound phage. The elute sample was diluted and infected host bacteria, and the bacteria culture was incubated at 37°C for 5 hours. This cycle of panning and recovery was repeated 3 more times, and single clones were subjected for sequencing. We have obtained one enriched clone expressing CITGSQNPC peptide, although we need to evaluate this clone further by injecting into a mouse to test the organ preference.

Establishment of immortalized bone marrow cells

The primary culture of bone marrow cells with ECGF are dominated by endothelial cell-like population. However, if they are cultured without ECGF, large flat cells are clearly observed, and hematopoeitic cells reside on the top of these large cells, suggesting our primary cultures contain stromal cells. In order to obtain both stromal and endothelial populations that may interact with breast cancer cells, we have made immortalized cell clones. Bone marrow tissue was collected from tibias and femora of four 10-week-old female Immortomice (Charles River, MA) and digested with collagenase A. The Immortomouse is a transgenic mouse carrying temperature sensitive SV40 large T antigen under control of the mouse major histocompatibility complex $H\text{-}2K^b$ promoter. One half of the bone marrow cells was cultured without ECGF, while the other half was cultured with ECGF to obtain endothelial cell clones. All the cells were maintained with 100 units/ ml γ-interferon in order to activate the $H\text{-}2K^b$ promoter, and incubated at 33°C which was permissive condition for the temperature sensitive large T antigen. After two passages, single cloning was performed by limiting dilution. Four to five weeks later, six clones cultured without ECGF (stromal cell group) and five clones cultured with ECGF (endothelial cell group) were trypsinized and expanded.

All clones were tested for expression of alkaline phosphatase, a marker of both endothelial cells and stromal cells, and all the five from stromal cell group and two endothelial cell group were positive (Fig. a, e). Two clones from stromal group, BMS3 and BMS6, showed binding to the MDA-MB-231 (Fig. b), while only one clone from endothelial cell group, BME5, showed some binding to the MDA-MB-231 cells (Fig. f). Stromal clone BMS6 contained both small cells and large flat cells (Fig. c), and showed spontaneous differentiation to adipocytes when they were kept for three weeks without passage (Fig. d), suggesting that these cells at least have the ability to differentiate into the mesenchymal lineage. Endothelial clone BME5 was stained for a marker of endothelial cells, von Willebrand factor, with immunohistochemistry, and this clone expressed von Willebrand factor when cultured either with or without ECGF (Fig. g, h).

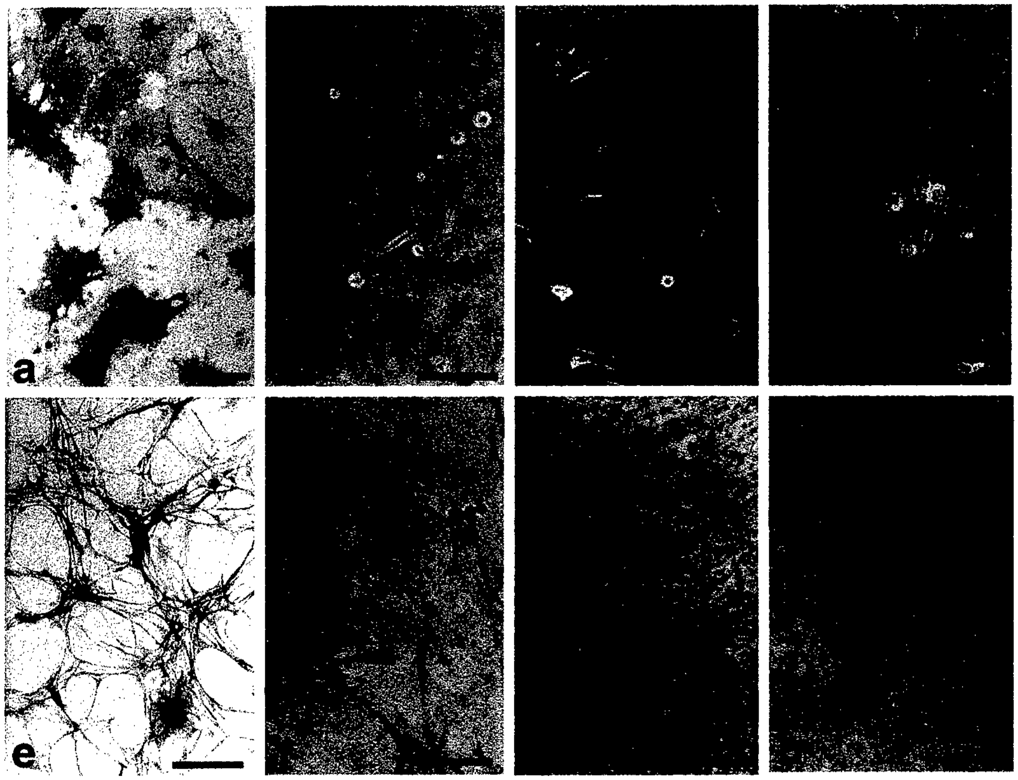

Fig. **(a)** and **(e)**: Alkaline phosphatase activity is shown in purple red. Nucleus are stained with methyl green. **(b)** and **(f)**: Disk assay. Nucleus of fixed BMS6 or BME5 cells on the disk are stained with trypan blue and bound live MDA-MB-231 cells appear as round shining cells (arrowheads). **(c)** and **(d):** phase contrast view of BMS6. Arrows show adipocytes. **(g)** and **(h):** localization of von Willebrand factor was shown in brown. BME5 showed flat morphology when cultured without ECGF **(h)**. **(a)**, **(b)**, **(c)** and **(d)** are clone BMS6. **(e)**, **(f)**, **(g)** and **(h)** are clone BME5. Bar=100 μm

7. KEY RESEARCH ACCOMPLISHMENTS

- Identification of 10 different peptide motifs that home to bone marrow by phage display experiments in vivo.
- Identification of one peptide motif by panning in vitro
- Eleven different immortalized bone marrow cell lines have been obtained.

8. REPORTABLE OUTCOMES

None at this time.

9. CONCLUSIONS

The identified motifs will be characterized for their ability to target bone marrow tissue and cells specifically. We will use the immortalized bone marrow cell clones to identify additional peptide motifs by in vitro panning as well as to attempt to clone cDNAs by expression cloning using subtracted cDNA libraries from metastatic and non-metastatic breast cancer cell lines. We expect to obtain bone-homing protein(s) that will confer bone localization when placed on the surface of a previously non-metastatic breast cancer cell line. This research will bring us much closer to finding a therapeutic inhibitor of metastasis and thus being able to block this most lethal phase of breast cancer.

10. REFERENCES

None

11. APPENDICES

None at this time